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NUCLEAR NONHISTONE PROTEINS IN MURINE MELANOMA CELLS: II. CHANGES FOLLOWING EXPOSURE TO MSH

MURIEL A. WIKSWO, PH.D., JOSEPH MCGUIRE, M.D., JANET E. SHANSKY, M.S., AND ROGER A. BOSHES, M.D., PH.D.

Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut, U.S.A.

Murine melanoma cells provide an excellent system for studying the proposed role of nuclear nonhistone proteins (NHP's) as regulators of gene expression. Cloudman mouse melanoma cells (S91, NCTC 3960, CCL 53), grown in culture, are normally lightly pigmented, but in the presence of melanocyte stimulating hormone (MSH) show a large increase in melanin content. Cells were grown in medium with and without MSH and labeled with either ^{14}C- or ^{3}H-leucine, respectively. Following 48 hr of incubation, the cells were harvested, combined, and nuclei isolated. The NHPs were extracted from these nuclei in a series of steps which yielded 4 major fractions. Each fraction was further separated on DEAE cellulose columns into a total of 40 subfractions, each of which was electrophoresed on SDS gels. Each gel was sliced and counted and the $^{14}C/^{3}H$ ratio was determined for each slice. A number of differences in $^{14}C/^{3}H$ ratios were observed between the NHPs isolated from MSH-treated and control cells which reflect changes in the synthesis and/or transport of NHPs in MSH-treated cells.

Nuclear nonhistone proteins (NHPs) have been implicated in the control of genetic expression [1–15]. We have used murine melanoma cells to test the proposed role of NHPs as regulatory proteins since the phenotype of the cell can be converted from amelanotic to melanotic by melanocyte simulating hormone (MSH). Moreover, tyrosinase is the only enzyme thus far identified with the oxidation of tyrosine to melanin [16]. Accordingly, the cells from which the NHP's are being compared may differ only in the synthesis or activation of this single enzyme.

A Cloudman melanoma cell line was used which, although normally lightly pigmented, exhibits a large increase in tyrosinase activity and melanin content in the presence of MSH [17–19]. The effect of MSH on melanoma cells is mediated by adenosine 3′,5′ monophosphate (cAMP) [17–22], perhaps at the transcriptional level by a block in the synthesis of an inhibitor of tyrosinase [18]. We have examined the NHPs in the melanoma cell following tyrosinase activation by MSH.

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Reprint requests to: Dr. Muriel Wikswo Lambert, Department of Pathology, CMDNJ, New Jersey Medical School, 100 Bergen Street, Newark, New Jersey 07103.

Abbreviations:
MSH: melanocyte stimulating hormone
NHPs: nuclear nonhistone proteins

MATERIALS AND METHODS

Culture Methods

Mouse melanoma cells (Cloudman S91 NCTC 3960-CCL 53) were grown in 250 ml Falcon culture flasks at 37° in Ham's nutrient mix F-10 (GIBCO) supplemented with 2% fetal calf serum (GIBCO), 10% horse serum (GIBCO), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 1.2 mg/ml of sodium bicarbonate. Each flask was seeded with 1×10^6 cells. After 48 hr the flasks were divided into 2 groups. To 1 group was added fresh F-10 medium lacking thymidine and leucine and containing 0.66 mg/ml racemized MSH; 4 μCi/ml L-(U-^{14}C) leucine (2.14 mCi/mg (New England Nuclear Boston, Mass); and 0.7 μg/ml thymidine. To the other set was added F-10 medium without MSH including 8.1 μCi/ml L-(4, 5-^{3}H) leucine (238 mCi/mg) (New England Nuclear), and 1.3 μg/ml L-leucine. The final concentration of leucine in both groups was 2 μg/ml (15% of the concentration of leucine in unaltered Ham's F-10 mixture). Cells were incubated in this medium for 48 hours.

Isolation of Nuclei

Cells from each group of flasks were released from the substrate with Ca^{++} and Mg^{++} free Tyrode's solution containing 5 mM EDTA. The suspension was centrifuged for 5 minutes at 500 ×g. The pellets were washed twice in 0.14 M NaCl and then homogenized in H buffer (0.05 M tricine, pH 7.5; 0.02 M KCl; 5 mM $MgCl_2$; 0.35 M sucrose) to which was added 3 mg/ml L-leucine. The homogenates from the MSH and control groups were combined and centrifuged at 500 ×g for 10 min. The pellets were suspended in 2.2 M sucrose, 1.5 mM $CaCl_2$ and the suspension was centrifuged for 30 min at 30,000 ×g to sediment the nuclei [23].

Extraction and Fractionation of Nuclear Nonhistone Proteins

The extraction of NHPs from the purified nuclei was a modification [23] of the method of Spelsberg et al [27]. The nuclear pellets were homogenized in NHP_1 buffer (10 mM Na PO_4, pH 6; 2 M NaCl; 5 M urea; 1 mM Mg Cl_2; and 0.1% β-mercaptoethanol) and then centrifuged for 24 hr at 105,000 g. The supernatant fraction contained the first nonhistone protein fraction (NHP_1) plus histones. The pellet, which included the remaining nonhistone protein plus DNA was resuspended in NHP_2 buffer (2 mM Tris-HCl, pH 7.5) and dialyzed for 2 hr against the same solution. This suspension was then centrifuged at 800 g for 10 min to sediment the insoluble protein, NHP_2. The supernatant fraction, containing DNA with the remaining NHP's, was mixed with 2 vol of NHP_3 buffer (15 mM Tris, pH 8.5; 3.0 M NaCl; 7.5 M urea; 1.5 mM $NaHSO_3$; 1.5 mM EDTA; and 0.1% β-mercaptoethanol) and allowed to stand at −20°C for 2 to 3 hr. This suspension was then centrifuged for 36 hr at 105,000 g. The supernatant fraction contained NHP_3 and the pellet contained DNA bound to the remaining protein fraction, NHP_4. Separation of NHP_4 from DNA was based [23] on a method of Teng, Teng, and Allfrey [6]. The pellet, containing DNA and NHP_4 was suspended in 5 vol of 0.1 M Tris-HCl, pH 8.4; 0.01 M EDTA, and 0.14 M β-mercaptoethanol (Buffer A). This suspension was mixed with an equal volume of cold phenol saturated with Buffer A and allowed to stand for 18 hr at 4°C. The mixture was then briefly homogenized and centrifuged at 12,000 g for 10 min. The phenol phase was collected and dialyzed at 4°C against 0.1 M acetic acid containing 0.14 M β-mercaptoethanol with 1 change until the phenol phase was reduced to about one-third of its original volume. The phenol phase was removed and dialyzed against 0.05 M acetic acid, 9.0 M urea, 0.14 M β-mercaptoethanol for 22 to 24 hr at 13°C. Dialysis was contained for 2 hr in NHP_4 buffer (0.1 M Tris-HCl, pH 8.4; 8.6 urea; 0.01 M EDTA; and 0.14 M β-mercaptoethanol).

Each of the NHP fractions was subfractionated by column chromatography. NHP_1 was separated from the histones on Bio-Rex 70 (200–400 mesh, Na form, Bio-Rad Laboratories) [23]. The column was equilibrated with the running buffer which contained 0.1 M $NaPO_4$, pH 7.0; 6.0 M urea; 0.35 M guanidine hydrochloride (G-HCl); 0.1% β-mercaptoethanol the NHP_1 plus histone fraction was dialyzed against the running buffer and applied to the column. The nonadsorbed peak contained NHP_1. The histones were eluted from the column with 4 M G-HCl in the running buffer, followed by 7 M G-HCl.

NHP_1, NHP_2, and NHP_3 were each fractionated on DEAE-cellulose (DE-52, Whatman). The DEAE columns were equilibrated with 0.01 M tris, pH 8.5; 3.0 M urea, 0.1% β-mercaptoethanol. NHP_1, NHP_2, and NHP_3 were each dialyzed against the above buffer and applied to the column. The NHP's were eluted from the column with a series of 7 to 11 steps of increasing concentrations of NaCl from 0.05 to 0.5 M. Residual protein was recovered in steps of 2 M NaCl and 4 M and 7 M G-HCl. The columns were monitored by counting aliquots of each fraction in 10 ml Scintisol (Isolab, Inc., Akron, Ohio) on a Nuclear Chicago Mark II scintillation counter. The fractions were pooled and frozen at −70°C.

Gel Electrophoresis

The 40 NHP fractions were dialyzed against distilled water, lyophylized, and further fractionated on 10% polyacrylamide SDS gels, 90 × 6 mm [23]. The gels were frozen, sliced transversly into 1-mm sections, and placed in scintillation vials containing 8 ml of the following mixture: 20 ml 4 N NH_4OH; [100 ml NCS solubilizer (Amersham/Searle Co.); and 1000 ml Liquifluor-toluene (1:24) (New England Nuclear Corp.). ^3H and ^{14}C were measured simultaneously in a Nuclear Chicago Mark II Scintillation Counter. Counting efficiency was 41% for ^3H and 55% for ^{14}C. The data were corrected for background and spillover and the $^{14}C/^3H$ ratios calculated. The significant differences in ratio, indicated by arrows in the figure, were observed in duplicate or triplicate gels and in a duplicate experiment.

RESULTS

An eightfold increase in tyrosinase activity [24] was observed in the present set of experiments after the cells were exposed to MSH for 48 hr. During this period the cell number increased in the control group from 8×10^7 to 13×10^7 and in the MSH-treated group from 8×10^7 to 12.6×10^7.

The 4 NHP fractions (NHP_1–NHP_4) as well as histones plus basic proteins contained between 80 and 95% of the total nuclear protein in the following proportions: NHP_1, 30–40%; NHP_2, 10–20%; NHP_3, 3–5%; NHP_4, 0.3–0.4%; and histones plus basic proteins, 30–35%. When the proteins were electrophoresed on polyacrylamide gels several differences were found between the $^{14}C/^3H$ ratios of the NHPs from melanotic and amelanotic cells.*

In NHP_1 the 0.05 M NaCl fraction exhibited 2 regions where the $^{14}C/^3H$ ratio decreased corresponding to proteins of roughly 17,000 and 26,000 daltons (Fig 1*c*). A decrease in the $^{14}C/^3H$ ratio was also observed in the 65,000 molecular weight region of the proteins in the 0.075 M fraction (Fig 1*d*). Slight increases in the $^{14}/C^3H$ ratios were observed in 14,000 and 22,000 molecular weight proteins in the 0.10 fraction (Fig 1*e*). The $^{14}C/^3H$ ratios of the 0.15 M NaCl fraction (Fig 1*f*) and 0.35 M-2 M NaCl and 4 M-7M guanidine-HCl fractions were relatively invariant as exemplified by the 0.4 M NaCl fraction (Fig 1*g*).

In NHP_2 variation in the ratios was probably related to the low number of counts in the sample applied to the gel. A slight increase in $^{14}C/^3H$ ratio was observed in the class of 70,000 dalton proteins in the 4 M guanidine-HCl fraction (Fig 2*c*).

* The statistical significance of observed changes in $^{14}C/^3H$ ratios was evaluated in two ways. In the first method, the point which most extremely deviated from the mean was selected, and a value was calculated based on the sample size (60–80 slices), which would be significant in a one-tailed test of significance. At the 1% level, this value was 3.6 SD and at the 5% level, 3.2 SD. This calculation was taken from the adjusted significance test proposed by Cooper [25]. The second method for evaluating significance was based on an a posteriori probability model. The significance of an observed deviation from the mean was determined by estimating the probability of such a deviation occurring at the same point as well as in the same direction in duplicate gels to 0.02% for triplicate samples. In all figures, those variations in the ratios which were significant according to one or both tests are noted by *arrows*.

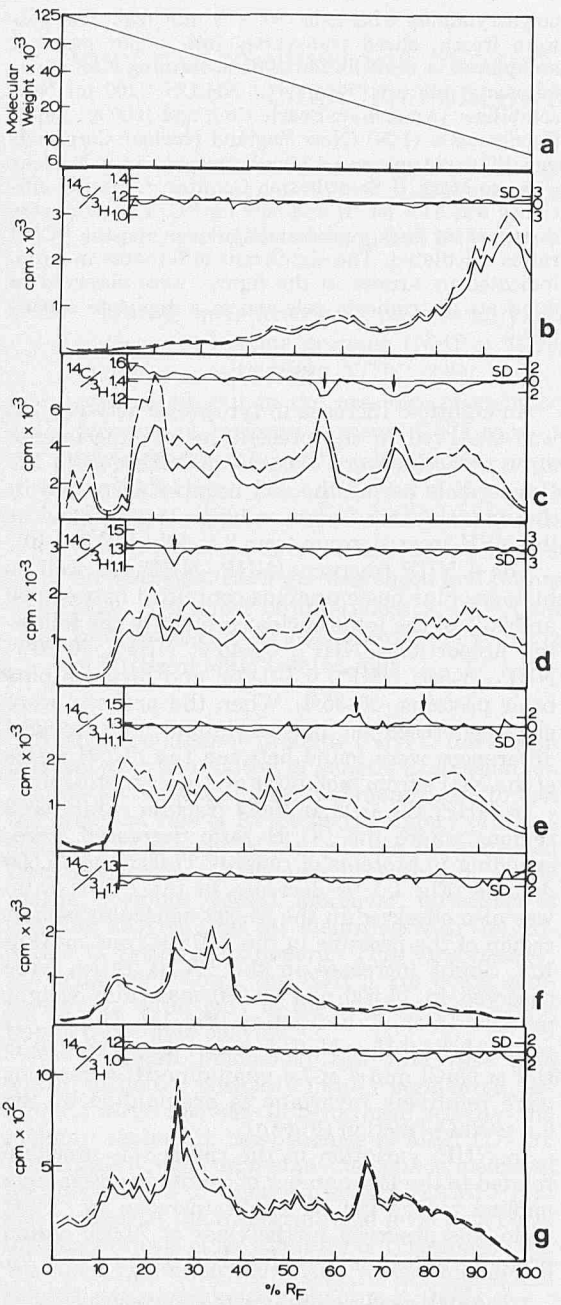

FIG 1. Changes in the labeling of NHP_1 fractions from MSH-treated melanoma cells. NHP_1 fractions were eluted from a DEAE-cellulose column with increasing salt concentrations as described in Materials and Methods. The pooled peaks were prepared and electrophoresed on SDS gels following the protocol of Teng et al [6]. Gels were sliced transversely into 1-mm sections and each slice was counted for 20 min. The data were corrected for background and ^{14}C spillover and the $^{14}C/^{3}H$ ratio was calculated for each slice. The mean and SD for each ratio are shown. Those variations in the ratios which are significant are noted by *arrows*. (*a*) molecular weight range of protein standards (see Materials and Methods). (*b*) unretained material (*c*) 0.05 M NaCl fraction (*d*) 0.075 M NaCl fraction (*e*) 0.10 M NaCl fraction (*f*) 0.15 M NaCl fraction (*g*) 0.4 M NaCl fraction. - - - = ^{14}C cpm (MSH-treated cells) and —— = ^{3}H cpm (control cells).

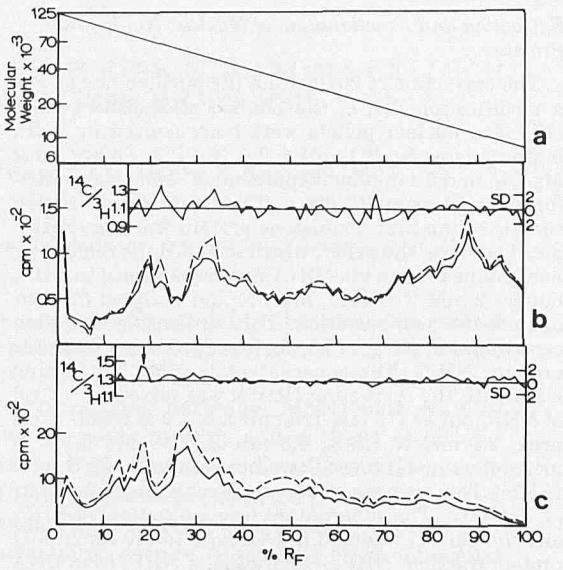

FIG 2. Changes in the labeling of NHP_2 fractions from MSH-treated melanoma cells. The fractions were eluted from a DEAE-cellulose column and electrophoresed on SDS gels. The protocol is the same as described in Fig 1. (*a*) molecular weight standards; (*b*) 0.5 M NaCl fraction; (*c*) 4.0 M G-HCl fraction; - - - = ^{14}C cpm (MSH-treated cells); —— = ^{3}H cpm.

In NHP_3 were found the major differences. In the 0.10 M, 0.125 M and 0.15 M fractions (Fig 3*b*-3*d*), there were several variations in the ratios. In particular, there was a large increase in the ^{14}C-labeled proteins of 20,000–22,000 daltons and smaller increases for ^{14}C-labeled proteins of 11,000 and 15,000 daltons. In the 0.125 M and 0.15 M fractions, increases in ^{14}C-labeled proteins of molecular weight 70,000 were also observed.

Since NHP_4 contained less protein than the other fractions, it was applied to SDS gels without prior subfractionation on DEAE. A large decrease in the ^{14}C-labeled proteins ranging from 10,000–16,000 daltons was observed (Fig 4*b*).

DISCUSSION

The alterations in the $^{14}C/^{3}H$ ratios reflect increases or decreases in synthesis and/or transport of particular NHP's between the cytoplasm and nucleus in the MSH-treated cells [26]. The most striking differences appear in NHP_3 where there are increases in NHP's of 20,000 and 70,000 daltons from MSH-treated cells. Spelsberg et al [27] have also found that in chick oviduct, the AP_3 fraction (which corresponds to NHP_3) contains proteins responsible for binding the progesterone-receptor complex to the chromatin. In other systems as well, the synthesis of NHP's is selectively stimulated by hormones such as hydrocortisone [28] and estradiol [29] in hepatic and uterine tissue, respectively, and by phytohemagglutinin in lymphoid cells [30]. The variations in $^{14}C/^{3}H$ ratios reflect changes in NHP's which could be involved in the regulation of any of the steps leading to the synthesis of melanin. The alterations in NHP's

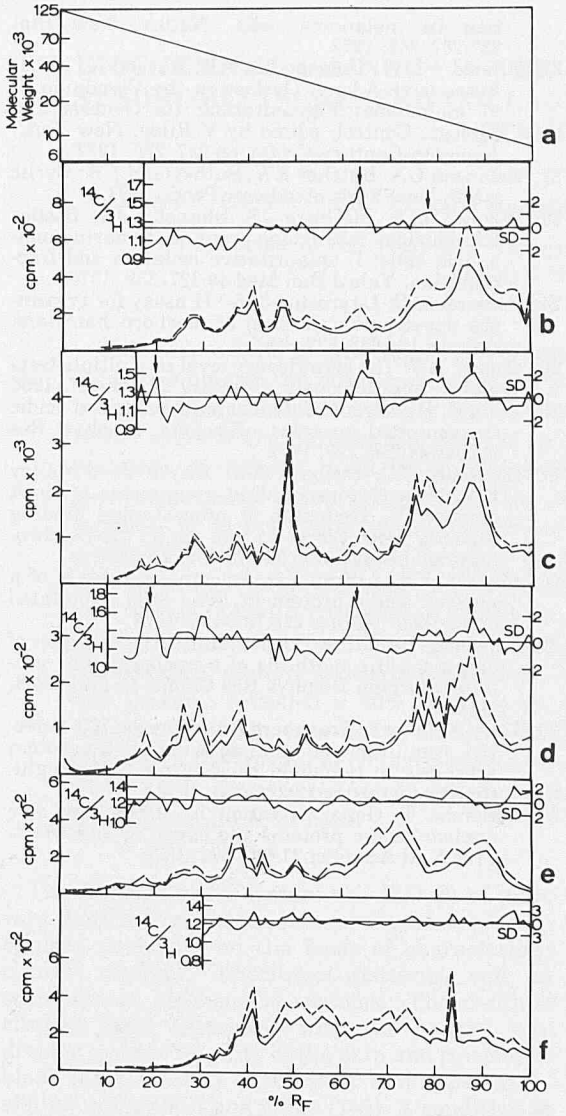

FIG 3. Changes in the labeling of NHP_3 fractions from MSH-treated melanoma cells. The fractions were eluted from a DEAE-cellulose column and electrophoresed on SDS gels. The protocol is the same as described in Fig 1. (*a*) molecular weight standards; (*b*) 0.100 M NaCl fraction; (*c*) 0.125 M NaCl fraction; (*d*) 0.150 M NaCl fraction; (*e*) 0.20 M NaCl fraction; (*f*) 4.0 M GHCl fraction; - - - = ^{14}C cpm (MSH-treated cells); —— = ^{3}H cpm (control cells).

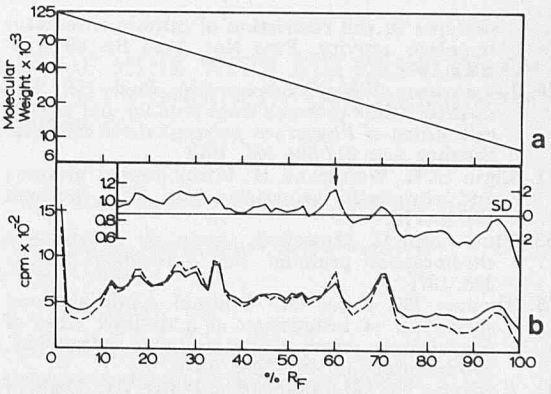

FIG 4. SDS gel of NHP_4 from melanoma cells treated with and without MSH. The sample was prepared and electrophoresed as in Fig 1. (*a*) molecular weight standards; (*b*) NHP_4 fraction; - - - = ^{14}C cpm (MSH-treated cells); —— = ^{3}H cpm (control cells).

could also reflect metabolic or morphological changes caused by increased intracellular cAMP [18,22]. The observed changes in NHP's in MSH-treated cells could be either a cause or a consequence of the response of the cells to the hormone [2,31].

By extensive subfractionation we have greatly increased the ability to detect small differences in specific NHP's. Total nuclear protein rather than chromatin was extracted to avoid losing soluble nonbound NHP's. Even with these improved methods for isolating and fractionating NHP's it is possible that changes in the amounts and types of proposed regulatory chromosomal proteins may escape detection. If a structural gene is represented by a nonreiterated sequence of DNA and has a single associated regulatory protein our method may not be sufficiently sensitive to detect a change associated with such a gene. Nonetheless, the analysis of melanoma cells which have such distinctive responses to hormones optimizes the identification of the hypothetical regulatory proteins within the population of NHP's. Recently, each of the 4 NHP fractions has been found to bind homologous DNA and influence template activity (Wikswo and McGuire, in preparation).

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